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Abstract: 1,2-Diarylethylamines represent a class of molecules that have shown potential in the treatment of pain, epilepsy, neurodegenerative disease and depression. Examples include lefetamine, remacemide, and lanicemine. Recently, several 1,2-diarylethylamines including the dissociatives diphenidine, methoxphenidine and ephenidine as well as the opioid MT-45, have appeared as 'research chemicals' or 'legal highs'. Due to their recent emergence little is known about their pharmacology. One of these, 1-[1-(2-fluorophenyl)-2-phenylethyl]pyrrolidine (fluorolintane, 2-F-DPPy), is available for purchase with purported dissociative effects intended to resemble phencyclidine (PCP) and ketamine. To better understand this emerging class, pharmacological investigations were undertaken for the first time on fluorolintane and its five aryl-fluorine-substituted isomers. In vitro binding studies revealed high affinity for N-methyl-D-aspartate (NMDA) receptors with fluorolintane (Ki = 87.92 nM) with lesser affinities for related compounds. Additional affinities were seen for all compounds at several sites including norepinephrine (NET), serotonin (SERT) and dopamine (DAT) transporters, and sigma receptors. Notably high affinities at DAT were observed, which were in most cases greater than NMDA receptor affinities. Additional functional and behavioral experiments show fluorolintane inhibited NMDA receptor-induced field excitatory postsynaptic potentials in rat hippocampal slices and inhibited long-term potentiation induced by theta-burst stimulation in rat hippocampal slices with potencies consistent with its NMDA receptor antagonism. Finally fluorolintane inhibited prepulse inhibition in rats, a measure of sensorimotor gating, with a median effective dose (ED50) of 13.3 mg/kg. These findings are consistent with anecdotal reports of dissociative effects of fluorolintane in humans.

Pharmacological characterizations of the legal high fluorolintane and isomers

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Abstract

1,2-Diarylethylamines represent a class of molecules that have shown potential in the treatment of pain, epilepsy, neurodegenerative disease and depression. Examples include lefetamine, remacemide, and lanicemine. Recently, several 1,2-diarylethylamines including the dissociatives diphenidine, methoxphenidine and ephenidine as well as the opioid MT-45, have appeared as 'research chemicals' or 'legal highs'. Due to their recent emergence little is known about their pharmacology. One of these, 1-[1-(2-fluorophenyl)-2-phenylethyl]pyrrolidine (fluorolintane, 2-F-DPPy), is available for purchase with purported dissociative effects intended to resemble phencyclidine (PCP) and ketamine. To better understand this emerging class, pharmacological investigations were undertaken for the first time on fluorolintane and its five aryl-fluorine-substituted isomers. *In vitro* binding studies revealed high affinity for *N*-methyl-D-aspartate (NMDA) receptors with fluorolintane (K_i = 87.92 nM) with lesser affinities for related compounds. Additional affinities were seen for all compounds at several sites including norepinephrine (NET), serotonin (SERT) and dopamine (DAT) transporters, and sigma receptors. Notably high affinities at DAT were observed, which were in most cases greater than NMDA receptor affinities. Additional functional and behavioral experiments show fluorolintane inhibited NMDA receptor-induced field excitatory postsynaptic potentials in rat hippocampal slices and inhibited long-term potentiation induced by theta-burst stimulation in rat hippocampal slices with potencies consistent with its NMDA receptor antagonism. Finally fluorolintane inhibited prepulse inhibition in rats, a measure of sensorimotor gating, with a median effective

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Keywords: NMDA receptor antagonist, legal high, fluorolintane, diphenidine, ketamine

1. Introduction

The past decade has witnessed an explosion in the number and availability of new psychoactive substances (NPS). These agents come from multiple pharmacological classes and are commonly produced to mimic the effects of classical psychoactive substances such as phytocannabinoids, psychostimulants, entactogens, dissociative anesthetics, sedative-hypnotics and hallucinogens while evading legislative restrictions. From the global perspective, the United Nations Office on Drugs and Crime (UNODC) reported the notification of 803 NPS in the period between 2009–2017 (UNODC, 2018). The European Monitoring Centre for Drugs and Drug Addiction (EMCDDA), responsible for collecting the information related to Europe, is currently monitoring over 670 substances that have emerged over the last 20 years (EMCDDA, 2018). One class of NPS are the dissociative drugs, which emerged as an effort to supply uncontrolled alternatives to the classical psychoactive drugs, PCP and ketamine (Morris and Wallach. 2014, Wallach and Brandt, 2018a, 2018b).

1-[1-(4-Methoxyphenyl)cyclohexyl]piperidine (4-MeO-PCP), is believed to be the first dissociative NPS, appearing around 2008. Other compounds soon followed with the earliest being structurally related to PCP (arylcyclohexylamines) and ketamine (*β*-ketoarylcyclohexylamines). Continuing legislative restrictions pushed the market to explore new chemical spaces including the 1,2-diarylethylamines such as diphenidine (Fig 1.). Subsequently, a number of 1,2-diarylethylamines have appeared, including methoxphenidine (MXP) and ephenidine (DPE) (Morris and Wallach, 2014). Analytical and pharmacological investigations of some of these compounds have been conducted (Wallach et al. 2015, Wallach et al. 2016, McLaughlin et al. 2016, Geyer et al. 2016, Luethi et al. 2017, Lodge and Mercier. 2015, Kang et al. 2017, Katselou et al. 2018). These 1,2-diarylethylamines were found to be potent *N*-methyl-D-aspartate (NMDA) receptor antagonists, with additional pharmacological effects including inhibition of monoamine transporters (Wallach et al. 2016, Luethi et al. 2017, Kang et al. 2017). The NMDA receptor is an ionotropic glutamate receptor believed to play a central role in the clinical and subjective effects of dissociative drugs in humans (Morris and Wallach. 2014, Lodge and Mercier. 2015, Ingram et al. 2018).

The pharmacology of several NPS 1,2-diarylethylamines remains completely unexplored. One such example is 1-[1-(2-fluorophenyl)-2-phenylethyl]pyrrolidine (fluorolintane, 2-F-DPPy). Anecdotal accounts of dissociative-type effects in humans have been reported on Internet discussion forums (e.g., UKchemicalresearch.org, 2015). To characterize the pharmacology of fluorolintane and related 1,2-diarylethylamines, experiments were conducted with fluorolintane, its five possible aryl-fluorinated isomers and the non-fluorinated counterpart, DPPy. Pharmacological investigations included NMDA receptor binding studies using competitive displacement of [^{3}H]MK-801 in rat forebrain, as well as binding studies at 40 additional CNS

sites, including receptors for serotonin, dopamine, norepinephrine, histamine, opioid and GABA, and transporters for serotonin, dopamine and norepinephrine. To investigate functional activity at NMDA receptors, the effects of fluorolintane on NMDA receptor dependent field excitatory postsynaptic potentials (fEPSPs) and on the induction of long-term potentiation (LTP) via theta-burst stimulation (TBS) was determined in rat CA1 hippocampal slices. Finally, experiments evaluated the effect of fluorolintane on pre-pulse inhibition (PPI) in rats, an operational measure of sensorimotor gating that is disrupted by dissociative drugs such as PCP and ketamine (Geyer et al. 2001, Halberstadt et al. 2016).

2. Materials & Methods

2.1 Test drugs

Fluorolintane, its five aryl-fluorine-positional isomers and DPPy were synthesized as hydrochloride salts as previously described (McLaughlin et al. 2016, Wallach et al. 2016). A full description of synthesis procedures and in-depth analytical characterizations has been published elsewhere (Dybek et al. 2019). All compounds were 95% pure or greater (HPLC, GC/MS and NMR).

2.2 NMDA receptor binding studies

In vitro binding affinities (K_i) of target compounds were determined by utilizing competitive radioligand binding assays with (+)-[3-^{3}H]-MK-801 ([^{3}H]MK-801, Perkin-Elmer, 15-30 Ci/mmol), in accordance with established protocols (Reynolds and Sharma 1999, 2001) similar to previous investigations (Wallach et al. 2016, Colestock et al. 2017, Kang et al. 2017). Rat forebrain homogenate was used as the source of NMDA receptors (rat brains obtained from Pel-Freez Biologicals, Rogers, AR, USA) and prepared as described by Reynolds and Sharma (1999). Suspensions of 10 mM HEPES buffer (pH 7.4) containing 100 μg/mL protein, 1.36 nM [^{3}H]MK-801, 100 μM glutamate, 10 μM glycine and seven concentrations (10^{-4}-10^{-10} M) of unlabeled test drugs as HCl salts dissolved in HEPEs buffer (competitors) were incubated in the dark on a mechanical rocker at room temperature for 2 h. (+)-MK-801 hydrogen maleate (30 μM; MedChem Express, USA) was used to define nonspecific binding. The reaction was terminated by vacuum filtration using a 24-well cell harvester (Brandel, Gaithersburg, MD, USA) over presoaked (15 min) GF/B glass fiber filters (Brandel, Gaithersburg, MD, USA). Filters were washed with room temperature HEPES buffer (15 mL per well). Tritium trapped on the filter was measured via liquid scintillation counting (Ultima Gold, Perkin Elmer), using a Beckman LS 6500 multipurpose scintillation counter (BeckmanCoulter, USA) at 57% efficiency. IC_{50} values were obtained by utilizing Graphpad Prism 5.0 (GraphPad Software, La Jolla, CA, USA) using nonlinear regression with log of the concentrations plotted against percent specific binding. K_i values were calculated using the equation of Cheng and Prusoff (1973). The K_d for (+)-MK-801 hydrogen maleate was determined to be 1.747 nM in previous studies. Protein concentrations were determined via the Bradford method using Coomassie protein assay reagent and rat albumin as standard (Fraction V, Sigma Aldrich, USA) (Bradford. 1976). Experiments were performed in duplicate and repeated three times. Finally (+)-MK-801 maleate (MedChem Express, USA) was also run (range 10^{-6}-10^{-11}, in duplicate, and experiments repeated three times) as a comparison control for this set of experiments.

2.3 Non-NMDA receptor binding studies

Competitive binding studies were conducted on fluorolintane and five isomers against 40 CNS receptors by the National Institute of Mental Health Psychoactive Drug Screening Program (NMH PDSP) using described methods (Besnard et al. 2012). Initially, target compounds were dissolved in dimethyl sulfoxide and screened for radioligand displacement against target receptors at 10,000 nM concentrations. Compounds showing >50% displacement of radioligand binding for a given protein target underwent secondary screenings at varying concentrations to determine K_i values. Compounds were run in triplicate on separate plates and each plate contained a known ligand of the receptor as a positive control. Full experimental details are available in the NIMH PDSP assay protocol book (Roth. 2018). A table of radioligands and receptors is provided in the supplemental document.

2.4 *In vitro* field excitatory postsynaptic potential (fEPSP) experiments

Male Wistar rats (Crl:Wi; Charles River, UK) aged 10 weeks were killed by neck vertebral dislocation (Schedule 1 method) according to the United Kingdom (Scientific Procedures) Act of 1986. Following rapid removal, brains were placed in artificial cerebrospinal fluid (aCSF) that consisted of 124 mM NaCl, 26 mM $NaHCO_3$, 3 mM KCl, 1.4 mM NaH_2PO4, 1 mM $MgSO_4$, 2 mM $CaCl_2$, and 10 mM D-glucose and were continuously oxygenated with 95% O_2 and 5% CO_2. Parasagittal cuts were made at 400 μm intervals and hippocampal slices were removed and placed in a submerged recording chamber at 28–30°C. Recordings of synaptic activity were then made, analyzed and presented as described (Ceolin et al. 2012). Using a recording glass microelectrode positioned in the stratum radiatum of area CA1, stimuli (0.03 Hz) were delivered with a bipolar electrode placed on the Schaffer collateral to elicit field excitatory synaptic potentials (fEPSPs). The NMDA receptor-mediated component of the fEPSP (NMDA receptor-fEPSP) was revealed by adding 10 μM NBQX, 50 μM picrotoxin and 1 μM CGP 55845 to the aCSF, which abolished AMPA and GABA receptor mediated transmission respectively. After 30 min of stable control responses, one concentration of the individual test compounds was added to the perfusate for 4-6 h while recording NMDA receptor-fEPSP on-line using WinLTP (Anderson and Collingridge. 2007).

For the LTP experiments slices were incubated for 2-3 h in fluorolintane (10 μM) before being transferred to the recording chamber. Once in the chamber the slices were continuously perfused with the drug until the end of the experiment. A theta burst stimulation (TBS) was delivered after 30 min of stable baseline. The TBS consisted of a single train of 5 bursts (each of 5 stimuli at 100 Hz) separated by 200 ms (Park et al. 2016). Control experiments were performed identically but slices were never exposed to fluorolintane.

2.5 *In vivo* pre-pulse inhibition (PPI) experiments

2.5.1 Animals

Male Sprague–Dawley rats from Harlan Industries (Indianapolis, IN, USA; initial weight 250–275 g) were housed in pairs under a 12 h reverse light/dark cycle (lights off at 0700 h). A reversed light/dark cycle was used so that behavioral testing could be conducted during the animals' awake phase. Food and water were available *ad libitum* except during behavioral testing. Animals were acclimatized for approximately 1 week after arrival prior to behavioral testing and maintained in Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) approved facilities that meet all federal and state guidelines. Procedures were approved by the University of California San Diego institutional animal care and use committee. Principles of laboratory animal care were followed as well as specific laws of the USA.

2.5.2 Apparatus

Eight startle chambers (SR-LAB system, San Diego Instruments, San Diego, CA, USA) were used to measure startle reactivity in rats (Mansbach et al. 1989, 1991). The startle test chambers are sound-attenuated, lighted, and ventilated enclosures containing a clear nonrestrictive cylindrical Plexiglas stabilimeter, 8.2 cm in diameter. A high-frequency loudspeaker mounted 24 cm above the Plexiglas cylinder produces all acoustic stimuli. The peak and average amplitudes of the startle response are detected by a piezoelectric accelerometer. At the onset of the startling stimulus, 100 x 1 ms readings were recorded, and the average amplitude used to determine the magnitude of the startle response (measured in arbitrary units). A dynamic calibration system was used to ensure comparable stabilimeter sensitivity across test chambers. Sound levels were measured using the dB(A) scale.

2.5.3 Acoustic startle test sessions

Acoustic startle test sessions consisted of startle trials (pulse-alone) and prepulse trials (prepulse + pulse). The pulse-alone trial consisted of a 40 ms 120 dB pulse of broadband white noise. Prepulse + pulse trials consisted of a 20 ms acoustic prepulse, an 80 ms delay, and then a 40 ms 120 dB startle pulse (100 ms onset–onset). There was an average of 15 s (range = 6–22 s) between trials. During each inter-trial interval, the movements of the animals were recorded once to measure responding when no stimulus was present (data not shown). Each startle session began with a 5 min acclimation period to a 65 dB broadband noise that was present continuously throughout the session. The startle test session contained 12 pulse-alone trials and 36 prepulse + pulse trials (12 prepulses each of 68, 71, and 77 dB [equivalent to 3, 6, and 12 dB above background]) presented in a pseudo-randomized order. Six pulse-alone trials were presented at the beginning and the end of the test session but were not used in the calculation of PPI values.

2.5.4 Experimental Design

Fluorolintane was dissolved in water containing 5% Tween-80 and administered subcutaneously. The injection volume was 1 mL/kg. One week after arrival, animals were tested in a brief baseline startle/PPI session to create treatment groups matched for levels of startle and PPI. On the testing day, rats were brought to the testing room and allowed to sit for

60 min before receiving injections. Animals (n=10/group, 40 total) were injected SC with vehicle or fluorolintane (3, 10, or 30 mg/kg) and then placed in the startle chambers 15 min later.

2.5.5 Analysis

The amount of PPI was calculated as a percentage score for each prepulse + pulse trial type: %PPI = 100−{[(startle response for prepulse+pulse trial)/(startle response for pulse-alone trial)]×100}. Startle magnitude was calculated as the average response to all of the pulse-alone trials. PPI data were analyzed with two-factor analysis of variance (ANOVA) with treatment as the between-subjects factor and trial type (prepulse intensity) as a repeated measure. For experiments in which there was no significant interaction between drug and prepulse intensity, PPI data were collapsed across prepulse intensity and average PPI was used as the main dependent measure. ED_{50} values were calculated using nonlinear regression (Prism 7.00, GraphPad Software, San Diego, CA). Startle magnitude data were analyzed with one-factor ANOVA. Post-hoc analyses were performed using Tukey's studentized range method. The alpha level was set at 0.05.

3. Results

3.1 NMDA receptor binding studies

Binding affinities for the test compounds and related compounds at the NMDA receptors are presented in Table 1. [^{3}H]MK-801 displacement curves for the seven 1,2-diphenylethylamines are included below in Fig 2. Of the seven compounds examined, five displayed moderate (10^{-7}-10^{-6}) NMDA receptor binding affinities (DPPy, 3-F-DPPy, 4-F-DPPy, 2"-F-DPPy and 3"-F-DPPy), whereas fluorolintane (2-F-DPPy) displayed a high affinity (below 10^{-7}) and 4"-F-DPPy had extremely weak affinity (>10^{-5}). The rank order of potency of the series evaluated here for [^{3}H]MK-801 displacement was found to be 2-F-DPPy > 3"-F-DPPy > DPPy > 2"-F-DPPy > 3-F-DPPy > 4-F-DPPy > 4"-F-DPPy.

Table 1. NMDA receptor binding affinities for fluorolintane, DPPy and positional fluorine isomers, and reference compounds using [^{3}H]MK-801 in rat forebrains. Means ± S.E.M from at least three separate experiments run in duplicate.

Compound	IC_{50} (nM)	K_i (nM)
PCP (Wallach 2014)	91 ± 1.3	57.9 ± 0.8
PCP (Colestock et al. 2017)	34.7 ± 2.5	22.1 ± 1.6
Diphenidine (Wallach et al. 2016)	28.6 ± 3.5	18.2 ± 2.2
Ketamine (Kang et al. 2017)	508.5 ± 30.1	323.9 ± 19.2
(+)-MK-801	4.5 ± 0.20	2.5 ± 0.11
(+)-MK-801 (Wallach et al. 2016)	4.1 ± 1.6	2.5 ± 1.0
Memantine (Wallach et al. 2016)	594.2 ± 41.3	378.4 ± 26.3
DPPy	498.7 ± 26.1	280.15 ± 14.67
Fluorolintane (2-F-DPPy)	156.5 ± 8.9	87.92 ± 5.03

3-F-DPPy	1,126.1 ± 119	632.64 ± 66.8
4-F-DPPy	1,697 ± 146	953.37 ± 82.5
2''-F-DPPy	1,085.33 ± 31.3	609.74 ± 17.6
3''-F-DPPy	466.1 ± 16.7	261.84 ± 9.4
4''-F-DPPy	18,300 ± 300	10,282.77 ± 169

3.3 Non-NMDA receptor interactions

All six fluorinated isomers showed affinities at several non-NMDA receptor CNS binding sites (Fig 3.). These sites included monoamine transporters for dopamine (DAT), serotonin (SERT) and norepinephrine (NET) as well as sigma-1 and sigma-2 receptors. This observation is consistent with that seen previously for diphenidine, 2-MXP and related compounds, as well as arylcyclohexylamines (Wallach. 2014, Wallach et al. 2016, Colestock et al. 2017, Kang et al. 2017, Luethi et al. 2018). 3-F-DPPy had the highest DAT affinity of the series (12 nM) whereas fluorolintane had the lowest (327 nM). All compounds except fluorolintane had higher affinity (K_i) at DAT than NMDA receptor. In the case of 3-F-DPPy, its affinity at DAT was ~52-fold higher than that for the NMDA receptor (632 nM). All compounds except fluorolintane and 4''-F-DPPy showed affinities for NET below 10,000 nM. Similar to DAT, the affinities at NET were generally greater than affinities for the NMDA receptor. Affinities at SERT were also detected though it was modest in most cases consistent with previous results on related compounds (Wallach et al. 2016, Luethi et al. 2018). Affinities for DAT were always greater than affinities at NET and SERT for a given compound. High to modest affinities for alpha2-adrenergic subtypes 2A, 2B and 2C were observed with most compounds. 4''-F-DPPy had the highest affinities for these three subtypes reaching 10^{-8}-10^{-7} potency. Diphenidine and methoxphenidine were found to have modest affinities at various alpha2 subtypes previously (Wallach et al. 2016, Luethi et al. 2018). None of the compounds showed affinity for the μ-opioid receptor (MOR), consistent with previous work with diphenidine, methoxphenidine, ephenidine and related compounds. Several of the compounds did however exhibit low to moderate affinities at the κ-opioid receptor (KOR), with the most potent being 2''-F-DPPy (K_i = 642 nM). (Wallach et al. 2016, Kang et al. 2016). This is of interest as MT-45 and lefetamine are 1,2-diarylethylamines which have been found to have opioid-like activity in various assays, including MOR and KOR affinities (Nakamura and Shimizu. 1976, Mannelli et al. 1989, Baumann et al. 2018, De Montis et al. 1985). Finally, affinities at sigma-1 and sigma-2 receptors were detected with all six F-DPPy isomers, except 4-F-DPPy which lacked affinity at the sigma-2 receptor (IC_{50} >10,000 nM). Affinities at the sigma-1 receptor were higher than the sigma-2 receptor for every compound. Sigma affinities were seen previously with diphenidine and related compounds (Wallach et al. 2016).

3.4 Effect on NMDA receptor-mediated field EPSPs.

Fig 4. shows the effects of test drugs and reference compounds on fEPSPs recorded from CA1 synapses in hippocampal slices following Schaffer collateral stimulation. In Fig 4., appropriate selective compounds were used to block the AMPA and GABA receptor-mediated

potentials and the stimulus strength was increased to provide a submaximal NMDA receptor-fEPSP (Kang et al. 2017). The lower case "c" displays the maximal effect of the competitive NMDA receptor antagonist, D-AP5 (100 μM). The graph shows the average fEPSP amplitude of 4-5 experiments expressed as a percentage of the baseline and after the perfusion of fluorolintane at concentrations of 1 μM (red), 3 μM, (blue) 10 μM (black).

As shown in Fig 4., 1 μM of fluorolintane reduced the NMDA receptor-fEPSP by 19.4 ± 3% after 90 min and 41.8 ± 4.3% after 240 min in a single experiment. The same pattern (Wilcoxon test, n=5, $p<0.05$, df=8) was observed in the pooled data of five different animals (mean ± S.E.M, n=5). To further probe this sensitivity of the NMDA receptor-fEPSP to fluorolintane, 3 μM and 10 μM fluorolintane were utilized in the same procedure. Effects on NMDA receptor-fEPSP were concentration dependent. The 3 μM concentration reduced the NMDA receptor-fEPSP significantly in a single experiment and within the pooled data by 37.1 ± 4%, after 90 min and 60.4 ± 3% after 240 min of drug perfusion (mean ± S.E.M, n=5, Wilcoxon test, $p<0.05$, df=8) 10 μM of fluorolintane produced a strong reduction/inhibition of NMDA receptor-fEPSP of 66 ± 4% after 90 min perfusion and reaching a near peak of 85.9 ± 1% after 240 min perfusion, as observed in a single experiment (Fig. 4) and in the pooled data (mean ± S.E.M, n=4, Wilcoxon test, $p<0.05$, df=8). Fig 4. displays the combination of the pooled data for each concentration suggesting an inhibitory concentration dependent effect of fluorolintane on NMDA receptor-fEPSPs.

3.5 Effect on LTP induced by TBS.

Previously we found that ephenidine blocks the induction of LTP induced by a standard Theta Burst Stimulation (TBS) protocol (Kang et al 2017), therefore, we next explored the ability of fluorolintane to block this form of synaptic plasticity at the CA1 Schaffer collateral pathway (Fig 5.). Panel A and B show LTP elicited under control conditions and panel C and D show that LTP is blocked by fluorolintane (10 μM) in a fashion similar to that observed in the presence of D-AP5 (Bliss and Collingridge. 1993). Panels A and C represent single experiments whereas panels B and D show pooled data.

3.6 *In vivo* Pre-pulse Inhibition Experiments

Similar to other NMDA receptor antagonists (Halberstadt et al. 2016, Wallach et al. 2016), fluorolintane significantly reduced PPI ($F(3,36)=11.55$, $P<0.0001$). Although there was an interaction between treatment and prepulse intensity ($F(6,72)=2.82$, $P=0.0159$), the 30 mg/kg dose of fluorolintane reduced PPI at all three prepulse intensities ($P<0.01$, Tukey's test). Therefore, Fig 6. shows the PPI data collapsed across the three prepulse intensities. The dose of fluorolintane that produced a 50% reduction of average PPI (ED_{50}) was 13.3 mg/kg (95% CI = 8.8–20.0 mg/kg). There was a significant main effect of prepulse intensity ($F(2,72)=66.36$, $P<0.0001$). Fluorolintane did not alter the magnitude of the startle response to 120 dB acoustic stimuli (Main effect: $F(3,36)=0.69$, *NS*; Table 2).

Table 2. Effect of fluorolintane on startle magnitude

Fluorolintane dose (mg/kg)	*N*	Startle Magnitude (mean ±

		S.E.M)
0	10	186.18 ± 18.64
3	10	235.10 ± 24.46
10	10	161.53 ± 42.70
30	10	195.28 ± 51.80

4. Discussion

It is unknown what site on NMDA receptors, 1,2-diarylethylamines bind to, however given structural overlap with PCP (benzylamine moiety) and MK-801 (which can be viewed as a conformationally restrained 1,2-diarylethylamine), which bind to the PCP-binding site, it is probable that 1,2-diarylethylamines bind to the PCP-site labeled by [^{3}H]MK-801. An MK-801 bound heterotrimric NMDA receptor structure was recently solved using CRYO-EM showing the PCP-binding site inside the ion channel pore (Lu et al. 2017). Additionally, the kinetics of channel blockade by 1,2-diarylethylamines suggest an uncompetitive use-dependent block common with channel blockers like ketamine and PCP rather than a competitive block as seen with AP5 (Wallach et al. 2016, Kang et al. 2017). Finally, reasonable structure activity relationship (SAR) overlap has been seen with respect to arylcyclohexylamines and 1,2-diarylethylamines with respect to the benzylamine moiety (Wallach et al. 2016, Kang et al. 2017).

The compounds tested herein had lower NMDA receptor affinities than PCP, diphenidine and ephenidine. However, fluorolintane and 3”-F-DPPy had higher NMDA receptor affinities than ketamine and memantine (Wallach et al. 2016, Kang et al. 2017, Colestock et al. 2017). The relatively high NMDA receptor affinity of fluorolintane is especially intriguing given the reported high affinity of 2-chlorodiphenidine (2-Cl-DPP), another *ortho*–halogen-substituted 1,2-diarylethylamine (Wallach et al. 2016, Gray and Cheng. 1989). Thus, 2-F or 2-Cl-substituions appear to increase affinity over the unsubstituted parent compounds.

Consistency in the SAR for methoxy-substituted diphenidine analogs and their PCP counterparts for NMDA receptor affinity was reported (3-MeO- > 2-MeO- > 4-MeO-) (Wallach et al. 2016, Wallach. 2014, Gray and Cheng. 1989). Unfortunately little SAR information is available for fluorinated PCP analogs. Some information exists with the phenylcyclohexylamine (PCA) series and related arylcycloalkylamines (Thurkauf et al. 1990, Sun et al. 2014, Wallach. 2014). The SAR of the fluorolintane series for NMDA receptor affinity (2-F-DPPy > DPPy > 3-F-DPPy > 4-F-DPPy) did not match the PCA series (PCA > 3-F-PCA > 4-F-PCA > 2-F-PCA, determined using [^{3}H]TCP displacement in rat brain homogenates) (Thurkauf et al. 1990). In the fluorinated arylbicycloheptylamine series, the rank order [^{3}H]MK-801 displacement, rat forebrain) was H- > 4-F ≥ 3-F > 2-F (Wallach. 2014). For arylcycloheptylamines fluorine substitution reduced affinity relative to the unsubstituted and a 3-F-substituent resulted in higher NMDA receptor affinity than 4-F (Sun et al. 2014), consistent with the PCA series (Thurkauf et al. 1990). These results suggest some general SAR differences between the various benzylamine containing NMDA receptor antagonists, which may be helpful for selecting unique polypharmacology or pharmacokinetics. The NMDA receptor affinity for DPPy was reduced at least an order of

magnitude relative to diphenidine. In general, PCP and PCPy and their aryl-substituted counterparts were found to have more or less comparable NMDA receptor affinities (Wallach et al. 2014). Low affinity at the NMDA receptor with DPPy relative to diphenidine was reported previously (Gray and Cheng. 1989).

So far, the SAR of fluorinated arylcycloalkylamines for the NMDA receptor and monoamine transporters has not been systematically investigated although some information is available. 3-F-PCP had modest NMDA receptor affinity (K_i = 1,150 nM), DAT (K_i = 891 nM) and NET (K_i = 1,297 nM), but not SERT (IC_{50} >10,000 nM) (Wallach. 2014). Interestingly, the arylbicycloheptylamine, exo-1-[2-(3-fluorophenyl)bicyclo[2.2.1]heptan-2-yl]pyrrolidine (exo-3-F-PBCHPy) had high affinity for DAT (K_i = 93 nM) and SERT (K_i = 32 nM) and the 2- and 4-F isomers had high SERT affinity but lower DAT affinity. These fluorinated arylbicycloheptylamines all had low μM affinity for NET (Wallach. 2014).

Consistent with the current study, high affinities for sigma receptors have been observed with other arylcycloalkylamines and 1,2-diarylethylamines, including diphenidine, 2-MXP and ephenidine (Wallach. 2014, Colestock et al. 2017, Wallach et al. 2016, Kang et al. 2017). The relevance of this remains unclear, but sigma-1 has been hypothesized to mediate some of the actions of the NMDA receptor antagonist dextromethorphan (DXM) including some intracellular effects (Su et al. 2010, Nguyen et al. 2014). 4-F-DPPy had low affinity at 5-HT_{2A} (1,781 nM). PCP and ketamine were reported to have weak μM affinity at 5-HT_{2A} (Kapur and Seeman. 2002). Likewise, a recent study reported similar weak μM affinity for diphenidine and 2-MXP at 5-HT_{2A} (Luethi et al. 2018). The relevance of this is unknown, although the relatively weak affinity suggests it is unlikely to have a major impact on the pharmacological actions of these compounds.

Consistent with related compounds (Wallach et al. 2015, Wallach et al. 2016, Kang et al. 2017), the slow inhibitory kinetics of fluorolintane in the EPSP experiments are more comparable to PCP, ketamine and memantine (uncompetitive NMDA receptor antagonists), as opposed to the rapid kinetics of D-AP5 (a competitive NMDA receptor antagonist). The slow kinetics of the antagonism suggest a requirement for channel opening to allow fluorolintane access to the binding site in the pore region of the NMDA receptor. From these findings along with *in vitro* binding data, it can be concluded that fluorolintane appears to be a potent NMDA receptor antagonist, being more potent than ketamine and memantine *in vitro* but less so than PCP and diphenidine (Wallach et al. 2016).

Fluorolintane (10 μM) inhibited LTP formation induced by TBS in the Schaffer collateral CA1 pathway of rat hippocampal slices with a potency comparable to its inhibition of NMDA receptor dependent fEPSPs. NMDA receptor antagonists are well known to prevent the induction of LTP following high frequency stimulation patterns including TBS (Collingridge et al., 1983, Stringer and Guyenet, 1983, Larson and Lynch 1988, Davis et al. 1992). For example, it was previously found that ephenidine blocked TBS-induction of LTP at 10 μM under comparable conditions to those used here (Kang et al. 2017). Such disruption in LTP induction with fluorolintane (10 μM) suggests that the drug potentially can cause cognitive disruption and amnesia (Bliss and Collingridge, 1993) and associated synaptic changes may be linked with the behavioural deficits observed in the PPI experiments..

Both competitive and noncompetitive NMDA receptor antagonists produce dissociative effects, and hallucinations in humans (Domino 1992, Oye et al 1992, Herrling 1994; Steinberg et al

1994, Grotta et al., 1995, Giroux and Scatton, 1996, Yenari et al., 1998). PPI is reduced by competitive and noncompetitive NMDA receptor antagonists in rodents (Mansbach and Geyer, 1989, 1991, Bakshi et al., 1999, Depoortere et al., 1999, Halberstadt et al., 2016, Wallach et al., 2016). The ability of NMDA receptor antagonists to induce dissociative effects and hallucinations has been linked to reductions in subcortical gating, resulting in sensory flooding (Vollenweider and Geyer, 2001). Similarly, reductions in PPI may reflect an impairment of sensorimotor gating. Hence, the same information processing deficits contributing to the PPI disruption may be responsible for the dissociative and hallucinogenic effects of these compounds. The PPI data demonstrate that fluorolintane produces PCP- and ketamine-like behavioral effects in rodents. These results, however, do not address the mechanism-of-action for fluorolintane effects on PPI. Although NMDA receptor blockade is thought to underlie the PPI-disruptive effects produced by PCP and ketamine (Halberstadt et al. 2016), fluorolintane is not selective for NMDA receptors, meaning that other pharmacological mechanisms could potentially contribute to the effect on PPI. Follow-up studies are planned to address this question.

Fluorolintane inhibited PPI with modest potency, with an ED_{50} of 13.3 mg/kg. For comparison, diphenidine had an ED_{50} of 9.5 mg/kg (Wallach et al. 2016), whereas PCP and (*S*)-ketamine had ED_{50} values of 0.88 and 2.86 mg/kg respectively (Halberstadt et al. 2016). While NMDA receptor antagonist potency appears to correlate with PPI inhibition for some NMDA receptor antagonists including PCP and ketamine (Halberstadt et al. 2016), so far 1,2-diarylethylamines appear to be an exception. Interestingly, however, the reduced potency of 1,2-diarylethylamines in the PPI paradigm in rats is consistent with many reports that these compounds have reduced potency in humans compared to PCP (~ 10-fold consistent with the PPI potency difference) and related compounds with high NMDA receptor affinity. This observation indicates that PPI data in rats has utility as a predictive screen for dissociative effects and assessing potency of new dissociative drugs in humans.

The reason for the discrepancy between NMDAR affinity and PPI and potency in humans with 1,2-diarylethylamines may be due to pharmacokinetics (Wallach et al. 2016). For example, the tested 1,2-diarylethylamines may have: poor absorbance and distribution, high metabolic liability, or be substrates for efflux pumps. The estimated logP of many 1,2-diarylethylamines (diphenidine 5.05 cLogP ChemDraw Ultra) is high which could limit absorbance and distribution however PCP has a comparable LogP value (PCP: 5.1) suggesting this is unlikely the full story (Kamenka and Geneste. 1981). Metabolism studies have been performed on several 1,2-diarylethylamines (Wink et al. 2016, Elliot et al. 2015, Minakata et al. 2015) and show extensive metabolism, though biological elimination half-lives are unknown. Anecdotal reports from ingestion by humans, do not support large variability in route of administration (ruling out a first-pass effect) and reports of several h durations of dissociative effects are common (Wallach and Brandt. 2018b). These reports seem inconsistent with high metabolic liability. Efflux via the human Pgp transporter was evaluated for ephenidine and the closely related *N*-isopropyl-1,2-diphenylethylamine (DPiP). Ephenidine lacked substantial Pgp substrate activity at concentrations up to 500 μM, where DPiP had a K_m of 4.6 μM (Meyer et al. 2013). Other 1,2-diarylethylamines should be investigated at Pgp and other efflux transporters. Finally, unidentified pharmacodynamics differences such as NMDA receptor subtype selectivity, involvement of other receptors that influence potency of the PPI response and dissociative effects in humans.

5. Conclusion

Fluorolintane, its five possible aryl-F-positional isomers and DPPy were evaluated to characterize their pharmacology. Compounds had a range of affinities for the NMDA receptor as well as several additional receptors including DAT and NET monoamine transporters and sigma-1 and sigma-2 receptors. Rank order of potency for NMDA receptor affinity was found to be fluorolintane (2-F-DPPy) > 3”-F-DPPy > DPPy > 2”-F-DPPy > 3-F-DPPy > 4-F-DPPy > 4”-F-DPPy. The higher NMDA receptor affinity seen with 2-F-DPPy was unexpected based on current SAR knowledge with arylcycloalkylamines however is consistent with the high NMDA receptor affinity seen with the related 2-Cl-DPP. The high affinities observed for DAT was notable, with most compounds showing higher affinity at DAT than NMDA receptor. In fact, 3-F-DPPy had ~50-fold higher affinity at DAT than NMDA receptor. This trend suggests interesting polypharmacology to be investigated in future experiments. Fluorolintane showed concentration dependent inhibition of NMDA receptor-fEPSP consistent with its action as an NMDA receptor channel blocker. Fluorolintane also prevented the induction of LTP by TBS in hippocampal slices with potency consistent with its NMDA receptor blockage. Finally, fluorolintane significantly reduced PPI with an ED_{50} of 13.3 mg/kg in rats. These results are consistent with reports that fluorolintane acts as a dissociative drug in humans likely through antagonism of NMDA receptors.

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Figure Legends

Fig 1. Structures of known dissociatives (PCP, ketamine and diphenidine), fluorolintane (2-F-DPPy) and its five possible aryl-F-positional isomers.

Fig 2. Competitive binding curves for structural analogs of fluorolintane, DPPy and five fluorinated positional isomers using [^{3}H]MK-801 in rat forebrains. Each concentration run in duplicate and experiments repeated 3 times.

Fig 3. Receptor binding heat map for fluorolintane and five fluorinated positional isomers. Numbers provided are K_i values. Green colored box without number means IC_{50} was >10,000 nM based on primary experiment. White box means affinity not determined. Full descriptions of experimental conditions including full receptor name associated with each abbreviation, radioligand use and concentration used are provided in supplemental Table S1. Additional details for non-NMDA receptor binding experiments can be found in the NIMH PDSP assay protocol book (Roth. 2018)

Fig 4. Plots showing the time-course of inhibition of NMDA receptor-mediated fEPSPs by increasing concentrations of fluorolintane. The top inserts are raw sample traces showing typical field excitatory postsynaptic potentials (fEPSPs) extracted from one of the 3 μM experiments. The traces (a, b & c) were taken at the times indicated on the plots by the letters in blue. Each point in the plots represents the averaged amplitude of 4 successive evoked fEPSPs triggered at 30 s intervals and recorded in the CA1 region of the hippocampal slices. The data are expressed as percentage of the control (mean ± S.E.M) plotted against time. The bars above the graphs indicate the perfusion times for the different concentrations fluorolintane and D-AP5. Each plot shows pooled data from the number of experiments indicated in the lower left corner.

Fig 5. Fluorolintane blocks the induction of LTP elicited by TBS at CA1 hippocampal synapses. A & C show a single representative experiment and B & D show the polled results from 5 and 6 experiments respectively. The traces are averages of four successive fEPSP responses obtained at the times indicated (a & b) for the experiments illustrated in A & C. Graphs A & B show the results from 5 interleaved experiments under control conditions and graphs C & D show the results from 6 slices incubated in fluorolintane (10 μM). A & B show the induction of LTP by TBS delivered (arrow) in the absence of drug after obtaining 30 min of baseline. In contrast C & D show that TBS delivered (arrow) after 30 min of baseline on slices incubated for at least 3 h in fluorolintane (Fig 4.). The treatment with fluorolintane blocked the induction of LTP by TBS although there was a short post TBS potentiation. Data are presented as mean ± S.E.M.

Fig 6. Effect of fluorolintane on startle magnitude and prepulse inhibition of acoustic startle (PPI). A total of 40 rats were used in this experiment (n = 10/group). PPI values shown are averaged across the 3 prepulse intensities (mean ± S.E.M). *Significant difference compared with vehicle control, p<0.01 (Tukey's test).

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Figure 1

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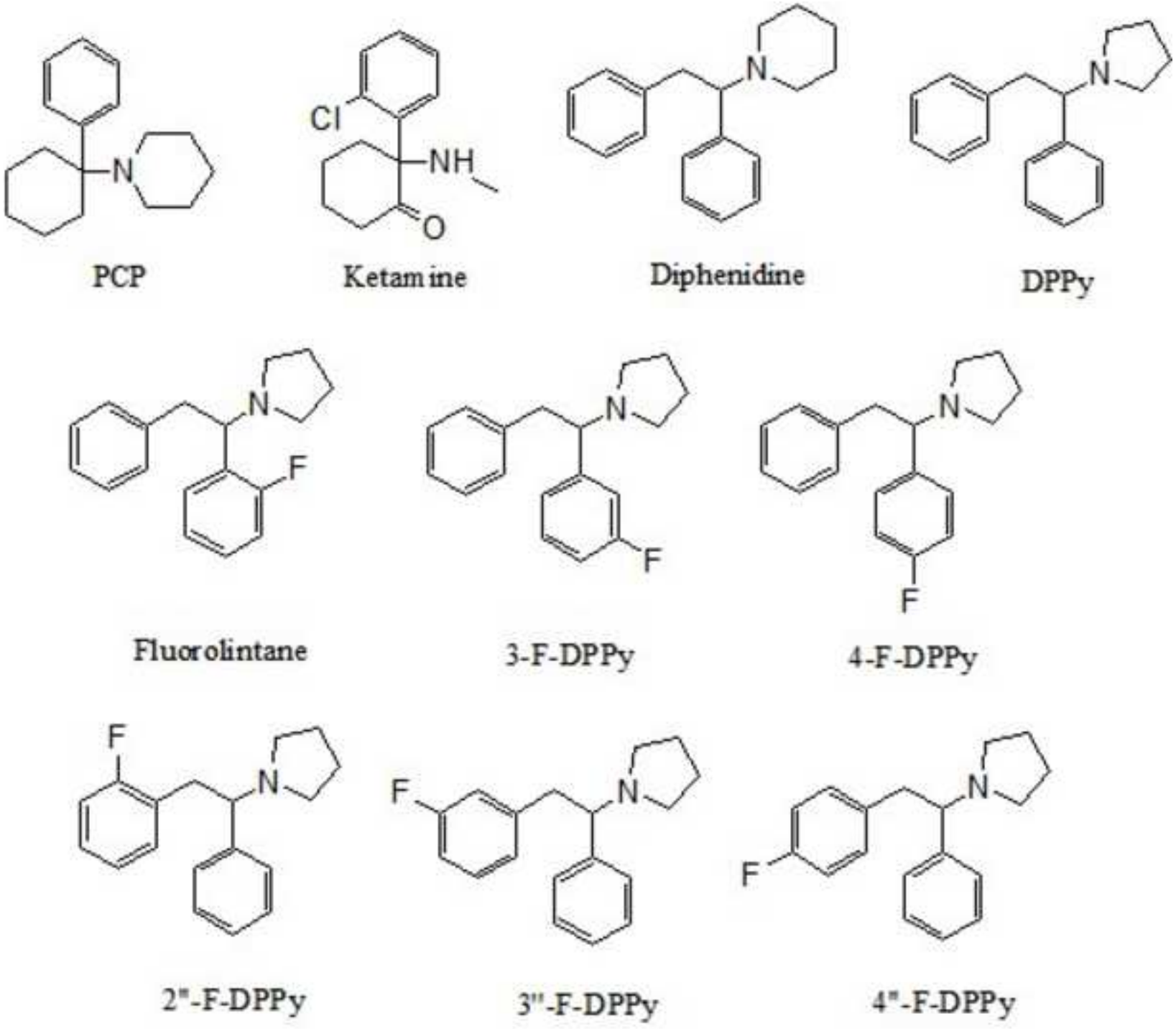

Figure 2

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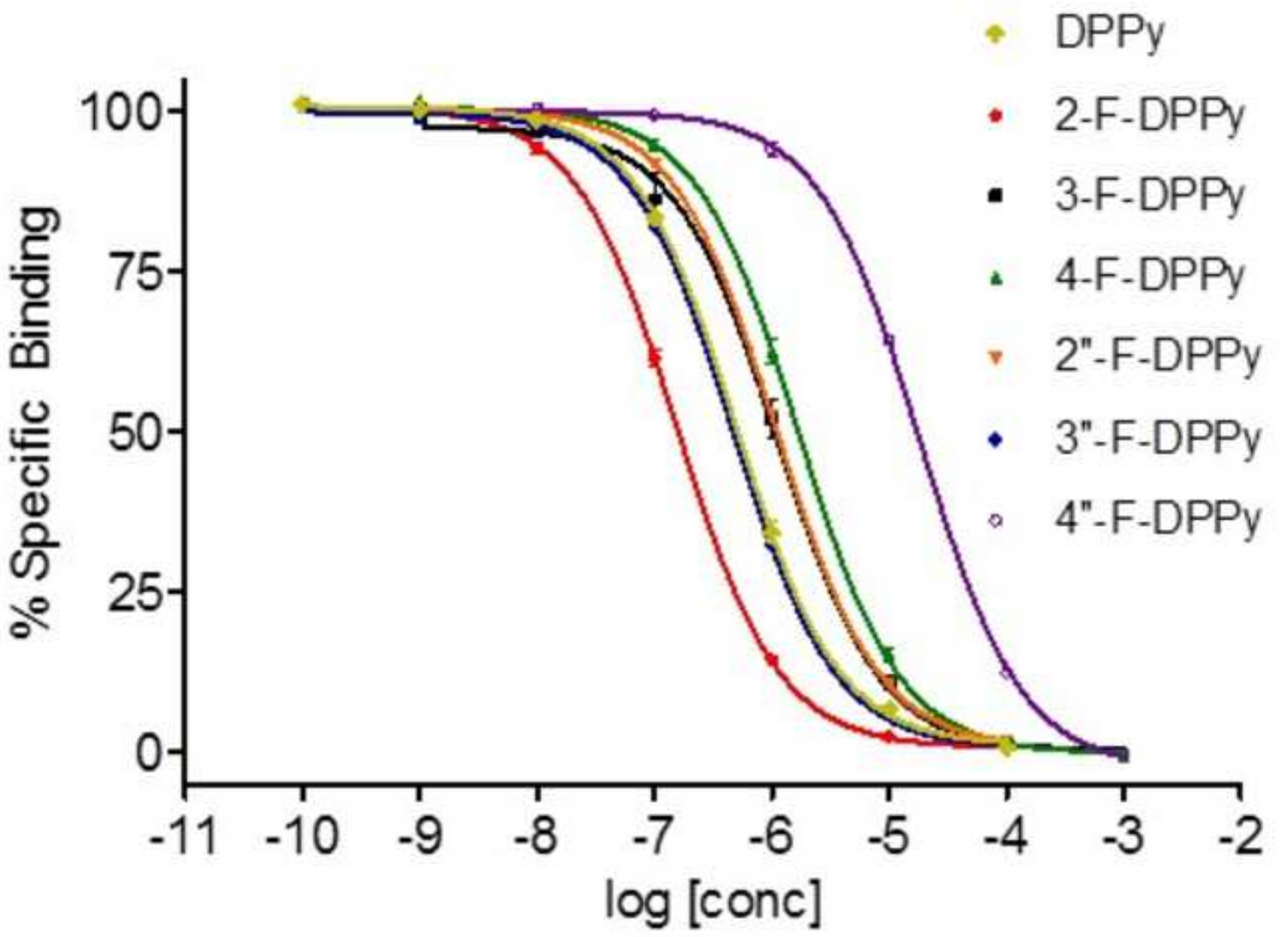

Figure 3

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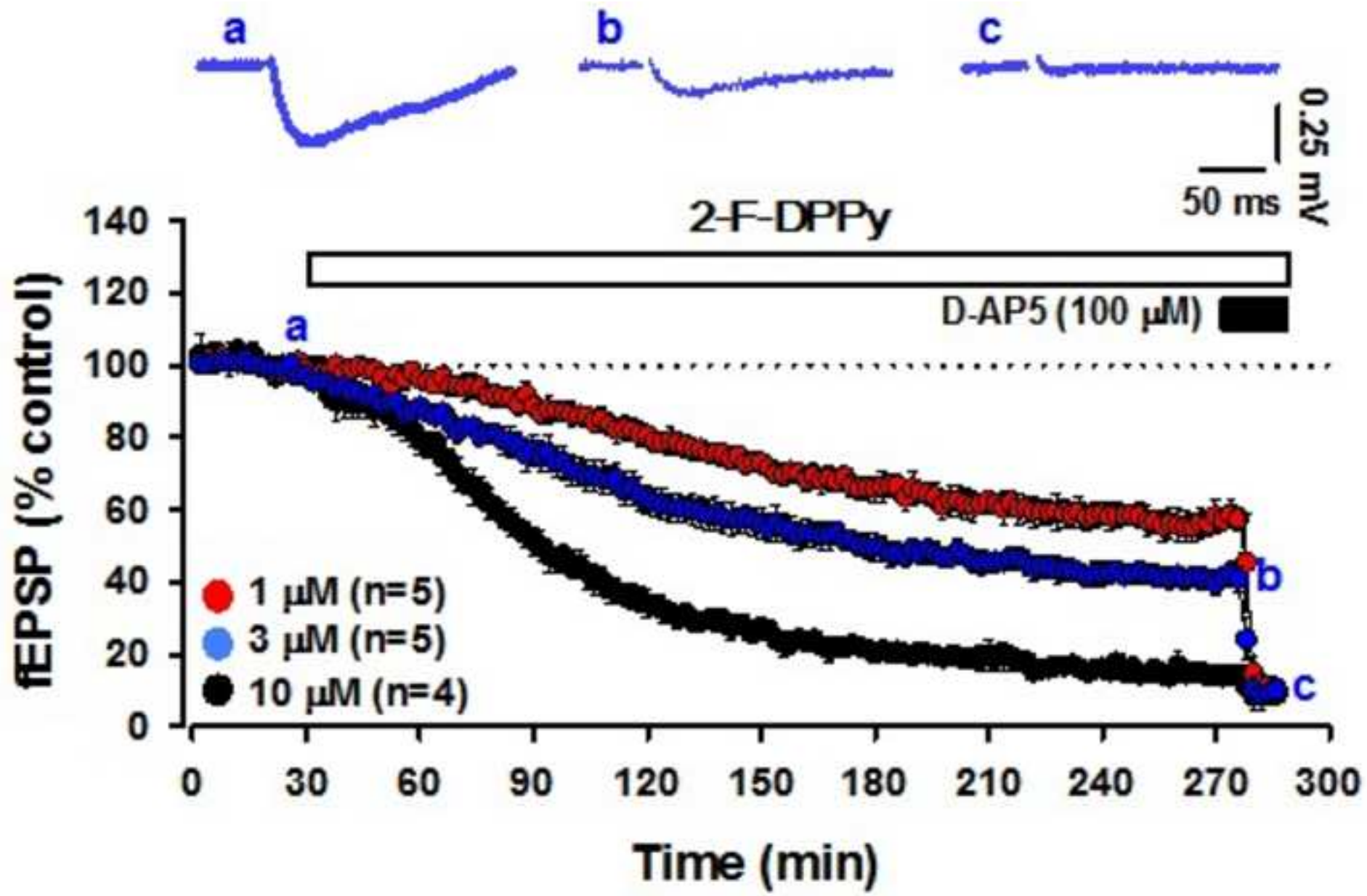

Figure 5

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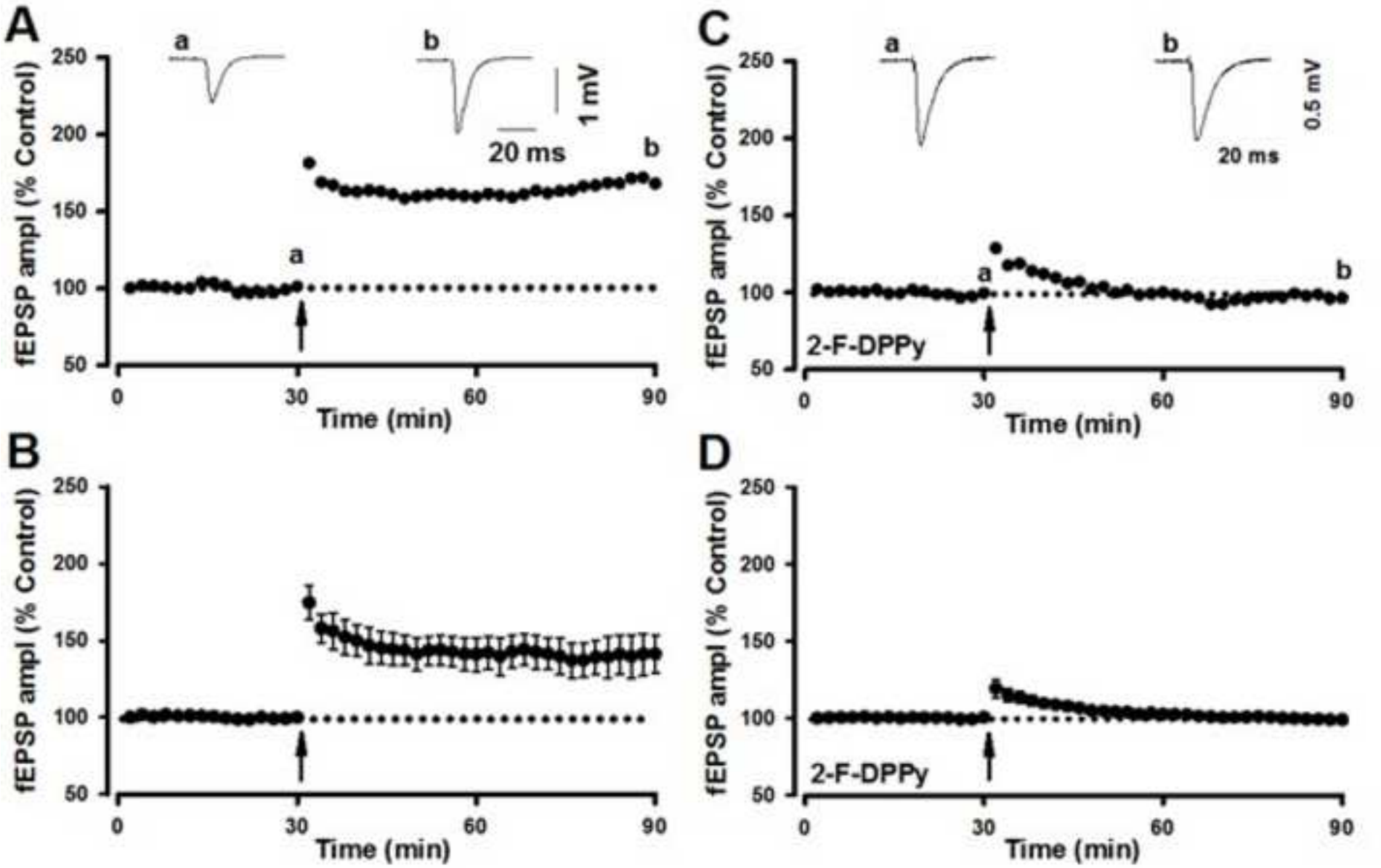

Figure 6
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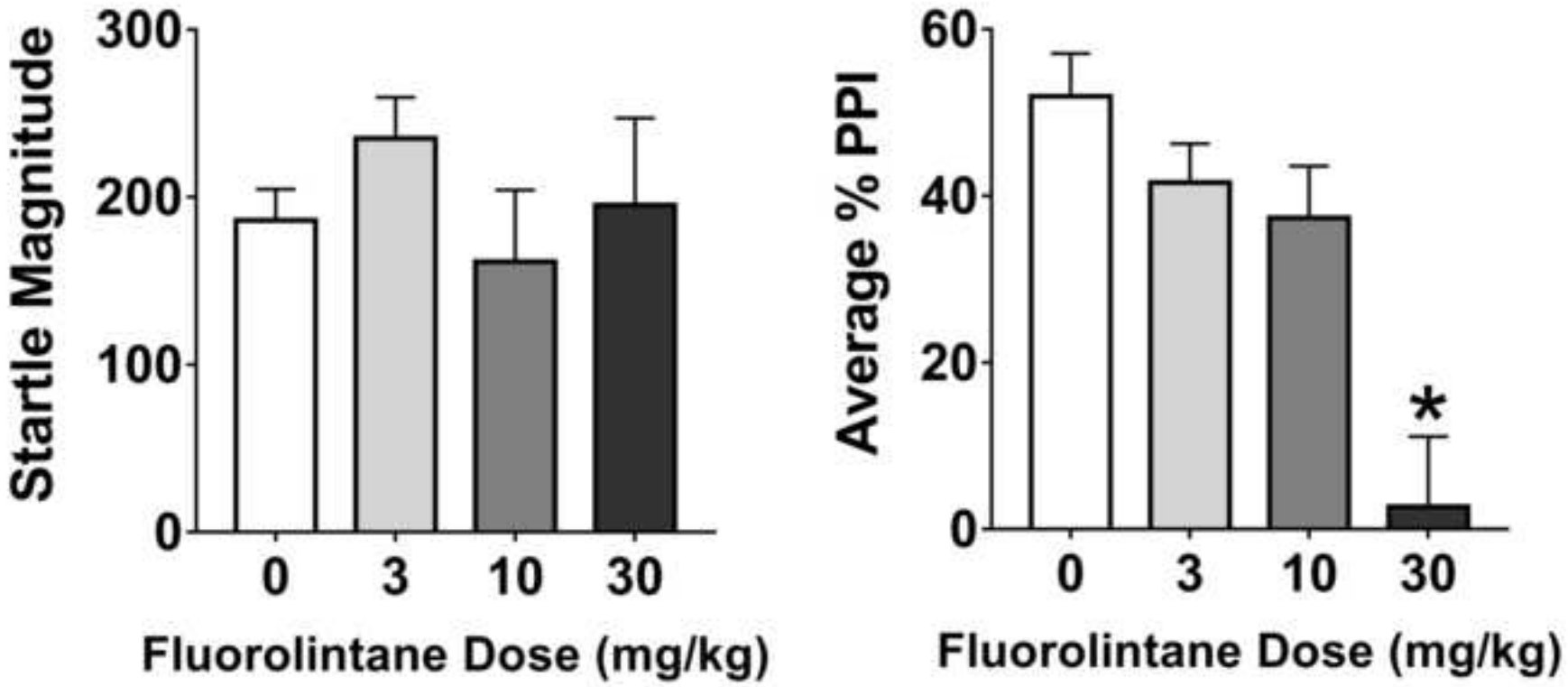

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Author Agreement

We declare no conflicts of interest.

Jason Wallach

***Response to Reviewers**

1. **Information of purity**

As stated by the authors, the compounds were synthesized in-house. The purity is an issue. Even the simple salts can affect much on the concentration and affinity to receptors. Authors need to provide more detail information.

Response: We have added additional details on the synthesis and analytical conformations of the target compounds to the methods section. The full syntheses and analytical characterizations of the target compounds will be the subject of a separate manuscript (which has just been accepted for publication in Drug Testing and Analysis with minor revisions requested). We feel that this is best suited for a separate manuscript (as we have done previously with related compounds) as the analytical portion is extensive and would be outside the scope of EJP. It would also add numerous additional authors. We expect the accepted analytical manuscript to be in production shortly and will add it as a reference once that occurs.

2. **Binding site of fluorolintane**

The Ki value of fluorolintane was estimated by the ligand binding assay with hot MK-801 (or other hot ligands for other receptor targets). This assay provide the information of the affinity. However, it does not provide any information about the binding sites. It was recommended NMR spectrometry or computer modeling can reveal the binding site more. However, it may be very demanding to request for this further experiment which needs a lot of time and technical input. Authors may like to elaborate more about the binding site.

Response: Excellent point and we agree that more detailed experiments to determine the binding site of 1,2-diarylethylamines on NMDA receptors are essential, though it is unfortunately outside the scope of our current capabilities. We are looking into this however as MK-801 bound NMDA receptors have recently been characterized using cryo-EM (Lu et al, Science, 335: 6331, 2017). That said we feel that given the compounds are structurally related to PCP (benzylamine moiety) and MK-801 (which can be thought of as a conformationally restrained 1,2-diarylethylamine), which are known to bind to the PCP binding site labeled by [^{3}H]MK-801. For this reason it is probable that it is this PCP-site where they bind. The kinetics of channel blockade seen is also consistent with an uncompetitive use-dependent blocker rather than a competitive blocker like AP5 (Kang et al, Neuropharmacology, 112, 2017, Wallach et al, PLOS One, 11(6):e0157021, 2016). We have expanded the discussion to address this important limitation and highlight the need for further studies.

3. **Validation of stimuli of PPI**

Authors has reported the effect of fluorolintane on the pre-pulse inhibition of rat with the setting of 120dB of pulse and 68, 71 and 77dB of prepulse. However, there is no information about the auditory response to this sound

intensity after the dosing of different concentration of compounds. i.e. the compound affect the auditory function itself. Did authors validate the experimental condition before running the test?

Response: The reviewer is correct that changes in auditory function could potentially affect the PPI data. However, the PPI experiments control for this potential confound by assessing the startle response induced by 120-dB acoustic stimuli. Manipulations that impair auditory function will also reduce the startle response. The fact that fluorolintane had no effect on the startle response indicates that auditory function was probably not affected by the drug.

4. **Other mechanism of PPI? Dopamine dependent or serotonin dependent mechanisms**
Fluorolintane was also with certain affinity to sigma 1 and 2 receptors and a relatively weak interaction with DAT according to authors' data. Is it possible the startle effect being modulated by affecting non-NMDA receptors?

Response: We added a paragraph to the discussion that focuses on this issue. NMDA receptor antagonists such as PCP and ketamine are known to disrupt PPI, an effect that is believed to have relevance to their dissociative and hallucinogenic effects. The PPI experiments were specifically conducted to determine whether fluorolintane mimics the effect of PCP and other dissociative drugs on PPI. Although the effects of PCP and ketamine on PPI are thought to be mediated by NMDA receptor blockade, it is important to consider that the PPI paradigm is not intended to serve as a specific behavioral readout of NMDA receptor antagonism, and it is possible that DAT or other targets could be involved in the effect of fluorolintane on PPI. Unfortunately, it is difficult to specifically investigate the pharmacological mechanism of action underlying NMDA receptor antagonist effects on PPI because multiple downstream transmitter systems may be involved in the effect. For example, although MK-801 is selective for NMDA receptors, MK-801-induced PPI disruption can be blocked by M100907 (Varty et al., NPP 20, 311, 1999), prazosin (Bakshi & Geyer, Neuroscience 92: 113, 1999), and the 5-HT1A antagonist WAY-100135 (Wedzony et al., NPP 23: 547, 2000).

5. **PPI vs electrophys data**
According to the electrophysiological data, a strong effect was induced on the LTP after the application of 10uM of compound (I presume it is equivalent to 30mg/kg in PPI experiment). The %PPI data showed that it is consistent. Furthermore, authors provided the ED50 in PPI is about 13.3mg/kg. These data indicated Fluorolintane has a high affinity to NMDAR but only a modest effect on PPI, suggesting other mechanisms may be involved. As also the authors claimed in discussion, the PK property may be a key to explain more and deeper, which is not available in this study. Authors need to discuss this point in details.

Response: We agree this is very interesting and have acknowledged it previously (Wallach et al, PLOS One, 11(6):e0157021, 2016, Wallach and Brandt. Handb Exp Pharmacol. 2018;252:305-352). We do plan to investigate this issue but the necessary experiments are more involved than the current project will allow. Most importantly though,
the PPI data seen with several 1,2-diarylethylamines has been consistent with the reported reduced potency of these compounds in humans to cause dissociative effects (relative to their high NMDAR affinities) supporting the utility of PPI to be predictive of dissociative potency of these compounds. We have added a paragraph to the discussion to address these important points.

6. **Other NMDAR related tests**
As stated in point 5, this compound may not work only on NMDAR. Authors may like to include some other behavioural tests involved NMDAR. Furthermore, in order to confirm the involvement of NMDAR (or other receptors), author may like to check the wash-out effect by applying the competitive ligands on one or some of the metrics. Again, that may need to have a detail PK and PD data to support.

We have used PPI because we have previously found it is predictive of potency of these compounds to cause dissociative effects in humans. As described above, under comment 4, additional mechanisms other than NMDA receptor antagonism can influence PPI. Thus PPI serves as a general test of a compounds potential to have dissociative effects in humans rather than an assessment of NMDAR antagonism potency. Importantly the results have been consistent with anecdotal reports of the potency of these compounds in humans (Wallach et al, PLOS One, 11(6):e0157021, 2016). As suggested additional models could be used to further characterize these compounds, specifically to address the role of NMDA receptors as well as downstream effects (elevated glutamate and monoamine release for example). The wash out effect is an interesting idea and we do plan to do more detailed behavioral studies in the future (such as locomotion studies), including detailed investigations on the role of NMDAR antagonism and its downstream effects on PPI. This has been clarified in the discussion.

The minor revision requests have also been made to the manuscript.

Cover Letter

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To the Editor,

We believe that the mechanistic investigations presented in our manuscript “Pharmacological Characterizations of the Legal High Fluorolintane and Isomers” are consistent with the goals and scope of The European Journal of Pharmacology and are honored for your consideration.

We have submitted a revised manuscript in which we have addressed the requested comments from the reviewers. Please see the response to the reviewer’s comments for specific details and responses. These comments were very helpful and we agree with them. By addressing them we have improved the clarity and relevance of the manuscript.

If you have any additional questions please do not hesitate to ask.

Thank you for your consideration,

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